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CHITIN, AN IMPORTANT NATURAL POLYMER

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There are many natural polysaccharides commercially available today. They include cellulose, dextran, pectin, alginic acid, agar-agar, agarose, starch, carrageenans and heparin; all of them are either neutral or acidic. Chitin and chitosan, the more or less acetylated polymers of glucosamine, on the other hand, are the only natural polysaccharides that have sharply basic characteristics, and, because of their basicity, they have unique properties: for example, their solubility in various media, the viscosity of their solutions, their polyelectrolyte behavior, membrane-forming ability and polyoxysalt formation. Their optical and structural properties are due to the presence of regularly spaced amino groups on the polyanhydroglucose chain.

From the chemical point of view, chitosan is a primary aliphatic polyamine, and therefore it can undergo all those reactions typical of amines. Its reactions with carbonyl compounds include acylation with acid anhydrides to form novel derivatives whose applicability ranges from selective aggregation of cancer cells to special coatings. Aldehydes react very easily with chitosan at room temperature to form Schiff bases. Products have been obtained from these bases that have unpredictable characteristics; again, their applicability ranges over a wide field, from the cross-linking of a tobacco sheet with chitosan and glyoxal, to enzyme immobilization on chitosan in the presence of glutaraldehyde. The analytical determinations of chitosan are today performed with either p-aminobenzaldehyde or 3-methylbenzo-2-thiazolone hydrazone. These determinations are sensitive enough to permit the detection of early stages of biodegradation by fungi of hydrocarbon fuels and measurements of fungal infiltration of cellulosic materials.

Chelation of transition metals, mostly a result of the presence of free amino groups, is offering new perspectives in chelation chromatography and in ligand-exchange chromatography. The establishment of quality standards for chitosan to be used in chromatography would certainly contribute to its wider applications as a chromatographic support.

Many ways of derivatizing chitosan are available; most of them involve hydroxyl groups. Sodium chitin, which has been known for many years, deserves more attention than it has so far received as a versatile compound. Carboxyl group formation, sulfation, cyanoethylation, glycolation, xanthation are just a few examples of the many reactions that can be carried out with chitin and chitosan. New products have been obtained from such reactions; semipermeable membranes for example, have been made with glycolchitosan, desalting has been carried out with formaldehyde cross-linked chitosan membranes and with regenerated chitin membranes, and the delayed release of drugs coated with glycolchitin has been studied *in vivo*. There is hope that blood anticoagulants can be produced based on chitosan that may operate in a manner similar to heparin.

The magnitude of the natural-resource base from which chitin can be obtained has been established in the United States, and estimates of production costs have been calculated. Industrial manufacture of chitin and chitosan appears to be feasible; available resources should, of course, not only be exploited, but also protected.

Many potential applications of chitin chemistry are of great industrial importance. Paper and textile additives and finishes, adsorbents for harmful metal ions, cements for leather manufacture, drilling muds, photographic products and coagulants useful for flocculating suspensions are some of the topics that have already been examined in the literature. Since chitosan is biodegradable, vegetable-canning waste suspensions coagulated with chitosan can safely be fed to animals.

In recent years, biochemical research has shown increasing interest in the biosynthesis of chitin and in the relevant enzymology. Chitin and chitosan are two of the polymers that provide structural support to many organisms. Fungal and animal chitin are not the only forms that occur: the polysaccharide from bacterial cell walls, composed of alternate N-acetylmuramic acid and N-acetyl-glucosamine units, can be regarded as an ether of lactic acid and chitin. Chitin is hydrolyzed by lysozyme. The biochemistry of lysozyme has been elucidated with the help of oligochitosaccharides.

The pathway of chitin synthesis has recently received important elucidation from investigations carried out *in vivo*; many other experiments have been done to study the synthesis of chitin *in vitro*. The roles of chitin synthetase and β-ecdysone are also under study. The purification of chitin synthetase and observations of the microfibrils that originate from chitosomes are still other recent accomplishments. Chitosomes have been described in terms of spheroidal organelles about 50 nm in diameter, which undergo a series of irreversible transformations when substrate and activator are combined to produce fibrils.

Chitin associations have received attention in recent years as well. For instance the concept of discrete layers of single components in fungi (α-glucan, protein, β-glucan and chitin) has been rejected in favor of structural gradients. Chitin deacetylase has been found in certain microorganisms where chitosan is produced through enzymatic deacetylation of chitin. The chitin biosynthesis can be inhibited by various substances such as polyoxins and insecticides derived from substituted ureas. Here again chitin assumes importance in our life as it plays a role in the agricultural sciences and in entomology. The insecticidal action of microorganisms (including viruses) is enhanced by chitinase. Associations of glucanase and chitinase as antimicrobial agents have been proposed for use in agriculture. There is evidence that the growth of the hyphal tips of fungi depends on a delicate balance between wall synthesis and wall lysis; chitinase can alter this balance.

Chitosanase has only recently been discovered; the purified enzyme hydrolyzes chitosan, but not chitin. It prevents spore germination of *Mucor* strains and causes a decrease in the turbidity of germinated spores of this fungus, which is known to contain chitosan.

In the light of available scientific and technical information, chitin appears today to be a substance of much greater significance and relevance than it seemed to have only a few years ago. The progress made in our knowledge of chitin also

provides an excellent example of the value of interdisciplinary research. Its implications for ecology, resource conservation, pollution prevention and agricultural and food-industry uses are obvious. Chitin seems to fulfill a number of demands in our technological world and, and at the same time, to be a key polymer for the preservation of our environment.

This First International Conference on Chitin and Chitosan was convened to affirm the importance of chitin and to integrate our expertise and knowledge in a common endeavor.

I. SOURCES OF CHITIN

DISTRIBUTION AND QUANTITATIVE IMPORTANCE OF CHITIN IN ANIMALS

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ABSTRACT

Using a specific and quantitative enzymatic-identification procedure, the polysaccharide chitin has been found in a wide range of animal species. It is used by protozoans, mainly ciliates, to build up cyst walls. It also constitutes the bulk of the stalks or stems of most hydrozoan colonies, but it is rarely produced by Scyphozoa (jellyfishes) and Anthozoa, and is absent in sponges.

Chitin is the main structural polysaccharide of most invertebrates belonging to the Protostomia. Arthropods are the best known and most important chitin-producing animals; the dry organic matter of their cuticles can contain up to 80% chitin. Besides the arthropods, relatively large amounts of chitin may be found in the setae of annelids (from 20 to 38% of dry weight), in the skeleton of the colonies of Bryozoa and in the shells and other structures (jaws, radulae, gastric shield) of many mollusc species (up to 7% of the dry organic matter in gastropods and bivalve shells, and up to 26% in cephalopods). Chitin is only absent in free and parasitic flatworms (Platyhelminthes), nemerteans, sipunculids and leeches. In some other groups, such as nematodes and rotifers, chitin is present only in the egg envelopes.

Chitin synthesis has never been observed in echinoderms or vertebrates, but the tubes of some Pogonophora contain 33% chitin, while tunicates secrete a chitinous peritrophic membrane.

From an ecological point of view, besides crustaceans and molluscs, marine benthic animals are a rich source of chitin. Despite their small size, bryozoan and hydrozoan colonies yield a large biomass with relatively substantial amounts of chitin. Some bryozoans play a role in the epuration of fresh water and produce a considerable biomass of chitin-containing colonies as well.

The proportion of "free" chitin, i.e., not bound to other organic molecules, is generally low (less than 10%), although it can account for 80% of the total chitin in mollusc shells.

INTRODUCTION

The first comprehensive studies of chitin distribution in animals (9, 10,20,26,28) were based on histochemical methods, such as the chitosan test by Campbell (5). These methods lacked specificity, however, and were sometimes unreliable, especially with small animals or when the amount of chitin was low (23,25). The more recent x-ray diffraction method (27) gives accurate but only qualitative results.

In order to obtain both qualitative and quantitative data on chitin

occurrence and localization, an enzymatic method based on the use of purified chitinases was developed (13,14). Owing to the specificity of chitinases (Enzyme Nomenclature:3.2.1.14) for the β-1,4 glucosidic linkages in N-acetylglucosamine polymers, this enzymatic method is highly specific for chitin, provided the purified chitinase preparation is devoid of other hydrolase. Moreover, this method enables us to discriminate the "free" chitin from the chitin chemically bound to other substances (14).

This distribution of chitin biosynthesis in animals has already been discussed from an evolutionary point of view (13,14,16). The aim of the present paper is to try to summarize the numerous data so far obtained with regard to the main ecological features of chitin-containing animals.

METHODS

Chitin was identified and estimated by the enzymatic method of Jeuniaux (13,14). After desiccation under vacuum, the material was weighed, treated with 0.5 N HCl at room temperature, washed, weighed, then treated with 0.5 N NaOH for 6 hours at 100°C. After washing, the residual material was suspended in a buffer (citric acid 0.2 M - Na_2HPO_4 0.4M) at pH 5.2 and incubated for 4-8 hours at 37°C with 1 ml purified chitinase (0.9 mg/ml for 0.02-2 mg chitin), using thymol as an antiseptic. After centrifugation, an aliquot of the supernatant was incubated with chitobiase (lobster serum 10 times diluted with distilled water) at pH 5.2 at 37°C for 2 hours. The liberated N-acetylglucosamine was determined by the method of Reissig et al. (24). The results are expressed as mg of chitin per 100 mg dry organic matter (chitin %).

"Free" chitin is estimated by the same procedure, omitting any previous treatment with NaOH.

The enzymatic method was also used for the qualitative detection of chitin in small animals (3,13,15). After treatment with HCl and NaOH, the washed residues were stained with Congo Red, then incubated with chitinase and observed under the light microscope.

Chitinase was purified from submerged culture filtrates of Streptomyces antibioticus (11) following the procedure described by Jeuniaux (12,13,15).

RESULTS

Micro- and meiofauna

Among protozoa, chitin is used by most ciliates to build cyst walls (14 of 22 species so far studied) (4), or sheaths in the case of the sessile species (Folliculina) that can sometimes be abundant on marine substrates.

The mesopsammic meiofauna pluricellular species living in soft sediments are mainly Turbellaria, nemerteans, nematodes, rotifers and gastrotrichs, which are devoid of chitinous structures. The eggs of nematodes, rotifers and gastrotrichs are, however, often provided with chitinoproteic envelopes (14.6% chitin with respect to total dry weight of the amictic eggs of Brachionus leydigii) (6,17).

Kinorhynchs, small and scarce mesopsammic marine animals, are covered with a chitinous cuticle. Tardigrades, which are often more common, also

possess(contrary to the opinion of some authors) a relatively thick cuticle made of chitin, probably bound to proteins (3).

Endoparasites

Chitin was found neither in parasitic flatworms (Cestoda, Trematoda) nor in round worms (Nematoda), except in the egg envelopes of the latter (16.6% in ascarid eggs) (13).

Terrestrial invertebrates

Chitin is the structural polysaccharide of the cuticle of insects, arachnids and myriapods (20 to 80%). Terrestrial tardigrades (3) and onychophorans (27) also possess a chitinous cuticle. The setae of earthworms (Oligochaeta) are also typical chitinous structures (27). In the pulmonate gastropods, snail shells contain small amounts (3%) of chitin (13), mainly as "free" chitin.

Planktonic and pelagic animals

The marine zooplankton are a rich source of chitin, being mainly formed by small holoplanktonic crustaceans and, in certain periods, by the meroplanktonic larvae of pelagic or benthic crustaceans. Their cuticular organic matter contains about 60-80% chitin.

Planktonic and pelagic coelenterates (Hydrozoa, Scyphozoa, Ctenophora) are devoid of chitin, with the exception of the pelagic colonies of Chondrophoridae such as Velella, the floating apparatus of which is a chitinous perisarc, as previously stated (10,22), containing about 48% chitin, almost entirely as "free" chitin.

The most important chitin-producing pelagic animals are the cephalopods, mainly cuttlefishes, the shells of which sometimes accumulate on the beaches with the tide. Chitin accounts for 26% of the organic matter of Sepia officinalis shells, and for 17.9% of squid (Loligo vulgaris) pens, mainly as "free" chitin.

Benthic marine fauna

Most benthic marine invertebrates produce chitinous structures, with the exception of sponges, flatworms (Turbellaria), nemerteans, echinoderms, sipunculids, pterobranchs and enteropneusts (7,13,27). The thick mantle, or tunic, of the sessile tunicates (sea squirts) is devoid of chitin, but these animals secrete a chitinous peritrophic membrane (21).

In crustaceans, the proportion of chitin in the cuticle is about 65-85% of the dry organic matter.

In molluscs, chitin was found in variable amounts in a wide variety of morphological structures (8,13,23), mainly as "free" chitin. The proportion of chitin varied from 0.1 to 7.3% in the periostracum, traces to 0.2% in prisms, 0.1 to 1.2% in mother of pearl, and from 0.2 to 8.3% in the calcitostracum of bivalve shells, the higher values being found in burrowing species such as Glycymeris, Venus, Pholas, Zyrphaea and Mya. The gastric shield of bivalves is also made of chitin (27.7% in Zyrphaea crispata [1]). In marine gastropods, the proportion of chitin was 3.0 to 7.0% in

mother of pearl of 3 species of Prosobranchia, 19.7% in the radulae, and 36.8% in the gizzard teeth of an opisthobranch. In the shell plates of Acanthochites discrepans (Polyplacophora), chitin amounted to 12%.

In different kinds of setae of marine worms (Polychaeta), chitin accounts for 20 to 38% of the dry organic matter and is mainly bound to quinone-tanned proteins. The tubes secreted by tubicolous worms do not contain any chitin.

Besides this macrofauna, benthic communities of the continental shelf also include the encrusting or erected colonies of hydrozoans and bryozoans (Ectoprocta). In both cases, the cuticular organic matrix of these colonies is made of chitin more or less bound to proteins, calcified in many species. In hydrozoans, the amount of chitin varied from 3.2 to 30.3% (13). In whole colonies of bryozoans (Flustra, Scrupocellaria, Cellaria, Crisia), the amount of chitin was 1.6 to 6.4%. Despite their small size, hydrozoan and bryozoan colonies may comprise an important element in epifaunal communities and may thus represent a large biomass of chitinous organic matter.

Chitin was also found in the stalk cuticle of Endoprocta, in the tubes of Phoronidea (13.5%), and in those of Pogonophora (33%) (2,7). The shells of brachiopods seemed to be devoid of chitin, with the exception of those of some Inarticulata (Lingula, Discinisca) (9), in which chitin amounted to 29% of the dry organic matter (13). The cuticle of the pedicle was chitinous in all the brachiopods so far studied (9,13,27).

Benthic freshwater invertebrates

Besides insects and crustaceans, a few bryozoan species may develop important colonies in some semi-polluted waters and give rise to the production of a large biomass (19) mainly made up of a chitinoproteic exoskeleton (ectocyst) (9,13). The biomass of Plumatella fungosa colonies in a pond was estimated to be 15.6 tons/ha (fresh weight) and the annual production to be 112 Kg nitrogen/ha/year. These colonies play a prominent part in the process of water purification (18).

CONCLUSIONS

If chitin is mainly secreted by cells of epidermal origin, the endoderm layer is also able to synthesize this polysaccharide, not only in arthropods, annelids and even tunicates (peritrophic membranes), but also in molluscs (gastric shields and gizzard plates).

The biosynthesis of chitin is a very old property of the animal cell, already present in Protozoa. This property was retained by most invertebrate animals of those groups belonging to the protostomian evolutionary lineage. At the top of this lineage, arthropods have exploited to a maximum the ability to use chitin as a structural polysaccharide, chitin often constituting, indeed, more than 50% of the cuticular organic matter. However, chitin may also be found in appreciable amounts in annelids, molluscs and in hydrozoan and bryozoan colonies, which form an important part of the marine benthic biomass.

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THE DISTRIBUTION AND QUANTITATIVE IMPORTANCE OF CHITIN IN FUNGI

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ABSTRACT

Chitin is the second most abundant organic compound on the earth, and fungi constitute its main source. Chitin is present in the vast majority of fungi as the principal fibrillar polymer of the cell wall. As such, it is responsible for the rigidity and the shape of the wall. Chitin is also present in the cytoplasm of some Oomycetes in the form of special granules (cellulin granules). The only major classes of fungi which lack chitin are the Schizomycetes, Myxomycetes and Trichomycetes. Those Oomycetes and Hyphochytridiomycetes which contain chitin also contain cellulose. Zygomycetes contain chitosan and chitin, whereas Ascomycetes, Basidiomycetes and Deuteromycetes, with the sole exception of yeasts, contain chitin as the only structural polymer. As a group, Euascomycetes are the fungi which contain the highest amounts of chitin, followed by Zygomycetes, Basidiomycetes and Deuteromycetes. Hemiascomycetes contain the lowest amounts of chitin. Highest values reported correspond to Allomyces macrogynus (58%) and Sclerotium rolfsii (61%). In general, conidia contain lower amounts of chitin than mycelium. In the case of dimorphic fungi, there is no correlation between cell shape and the relative amounts of chitin present in the cell wall.

INTRODUCTION

In 1811 Braconnot described a substance which he found in fungi and which he called "fungine" (31). It was not until the end of the century that the substance was rediscovered and identified as chitin, which had been known to be present in Arthropoda. X-ray diffraction studies have revealed that the degree of crystallinity of chitin from most fungi is similar to that present in invertebrates.

It was later demonstrated that chitin in fungi was located in the cell wall. Oomycetes belonging to the order Leptomitales contain, in the cytoplasm, special granules called cellulin granules. These granules were thought to be made of modified cellulose (28), but recent analyses of the genus Apodachlya have shown that they are made of β-1-3, β-1-6 glucans and chitin (20). These granules constitute the only exception where chitin is not present in the cell wall but in the form of a cytoplasmic inclusion.

Chitin is not the only compound present in the cell walls of fungi and, in fact, it may be a minor component. Other compounds present in the cell wall are cellulose, other polysaccharides, proteins and lipids.

Chemical composition of the cell walls of fungi

Two types of components can be recognized in the cell wall of fungi: structural components and amorphous components. Amorphous components comprise lipids, proteins and different types of polysaccharides. They are

important for the resistance of the wall and for the protection of the cell from different harmful compounds. Besides, some proteins of the cell wall have enzymatic activity. Structural components are responsible for the shape and rigidity of the cell wall. Using microchemical tests, van Wisselingh (40) recognized that fungi had either cellulosic or chitinous walls. Further refinement in the analytical methods sheds some doubts on the results obtained with microchemical reactions, but more recently it has become evident that fungi have chitin, cellulose and other β-glucans as their structural polymers.

Chitin is the sole structural component of fungal cell walls, where it is responsible for their shape and rigidity. As evidence for this statement, it can be shown after the removal of amorphous cell components from the cell that the cell wall maintains its original structure (33). Treatment of whole cells with chitinase seldom removes the cell wall or gives rise to protoplasts. This failure to hydrolyze the cell walls is due to the inaccessibility of the chitin to the enzyme. On the other hand, once the amorphous cell components are removed, chitin becomes accessible to chitinase, is hydrolyzed, and the shape of the cell wall is lost.

Particular fungi mutants, unable to synthesize chitin, form swollen hyphae and are osmotically sensitive (19).

Polyoxins, a group of antibiotics produced by Streptomyces cacoi, interfere with chitin biosynthesis (13). This antibiotic inhibits the growth of fungi (16,17), and at suboptimal concentrations induces morphological alterations of the cells (6,8).

Zygomycetes contain chitosan, besides chitin, in their cell wall. Apparently, in these fungi, chitosan plays an important role in the rigidity of the wall, since their cell walls are lysed by a chitosanase preparation obtained from Streptomyces sp.(29). This preparation does not lyse fungal chitin.

There is a strong correlation between the chemistry of the cell wall and the taxonomy of fungi. Bartnicki-Garcia (3) has distinguished 8 groups of fungi according to the chemistry of their cell walls (Table 1). These chemical groups coincide closely with accepted taxonomic and evolutionary concepts.

Chitin is present in most fungi and, according to this classification, it may be the only structural component (groups V and VII), or it may share this role with cellulose in group III and in some Oomycetes (22,23) not considered in Table 1, or with chitosan (group IV). In yeasts, chitin is only a minor component, and it is localized in the rim of the bud scars (10).

TABLE 1

CHEMOTYPES OF FUNGAL CELL WALLS*

	CHEMOTYPE	TAXONOMIC GROUP
I	Cellulose-glycogen	Acrasiales
II	Cellulose-glucan	Oomycetes**
III	Cellulose-chitin	Hyphochytridiomycetes
IV	Chitosan-chitin	Zygomycetes
V	Chitin-glucan	Chytridiomycetes
		Ascomycetes
		Basidiomycetes
		Deuteromycetes
VI	Mannan-glucan	Saccharomycetaceae
		Crytococcaceae
VII	Mannan-chitin	Sporobolomycetaceae
VIII	Polygalactosamine-galactan	Trichomycetes

*From Bartnicki-Garcia (3)
**Some members of the order Leptomytales have been shown to contain also chitin in the cell wall (22, 23).

METHODS

Detection of chitin in cell walls of fungi has been achieved by the classical microchemical test of van Wisselingh (40). This method may give erratic results, however, so its use has been mostly abandoned as a reliable test for chitin. Several authors have made use of the characteristic insolubility of chitin as a method of detection. These authors regard a compound which is insoluble in acid and alkali and releases glucosamine by hydrolysis with 6N HCl to be chitin. More reliable is the use of infrared spectroscopy to detect chitin. Michell and Scurfield (26) made a careful study of the infrared spectra of standard compounds and found that chitin was easily recognized from other cell wall components by its characteristic bands. They compared the spectra of isolated and extracted cell walls from several fungi with the standards and recognized the presence of chitin and cellulose in the different genera examined. Infrared spectroscopy has been used to detect the presence of chitin in Aspergillus (33), Morchella (35) and Choanephora cucurbitarum (21). The method par excellence to detect chitin is the use of x-ray diffraction. X-ray powder diagrams have been extensively used to detect chitin in whole cells, isolated cell walls and extracted cell walls.

Galun et al.(15) used an ingenious method to detect the presence of chitin in three fungi isolated from lichens. These fungi grow so meagerly that it was impossible to collect enough material to carry out chemical analysis. The authors regarded the incorporation of N-|^{3}H| acetyl glucosamine and the binding of fluorescein-conjugated wheat germ agglutinin as evidence for the presence of chitin in the cell walls of these lichen fungi.

Quantitative determination of chitin involves measurement of hexosamines in acid hydrolysates of the cell walls. Since during hydrolysis N-acetyl hexosamines are deacetylated, the method does not distinguish between polymers of hexosamines and N-acetyl hexosamines. Most authors first extract alkali-soluble glycoproteins and acid-soluble chitosan before measuring chitin. It is also important to determine whether all the hexosamine released is glucosamine or if there are also other hexosamines, of which galactosamine is the most abundant. A gentler and more specific method to determine chitin is the measurement of N-acetyl glucosamine released by enzymatic hydrolysis with chitinase and chitobiase. This method has been seldom used and then only for specific purposes (11).

Microfibrillar structure of the cell walls of fungi

Several fungi which contain chitin in their cell wall have been examined by electron microscopy. It is a general observation that the outer surface of the cell wall appears rather smooth, or at most granular, whereas the inner surface shows the presence of microfibrils. The microfibrillar appearance of the cell walls becomes more apparent when these are extracted with acid and alkali or treated with specific enzymes to remove amorphous compounds. Microfibrils have been observed by electron microscopy, mostly after shadowing, but also heavy metal replica have been prepared (21). Negative staining of cell walls from Histoplasma farciminosum (36) revealed the presence of microfibrils, both isolated and in bundles, measuring ca. 6 nm. Similar microfibrils 2-7 nm wide have been observed in oblique and tangential sections of Gilbertella persicaria (9).

In most fungi studied, microfibrils do not follow a particular orientation, but rather they are randomly oriented. Nevertheless, Scurfield (37) described that in the inner surface of Polyporus millitae microfibrils showed a strong tendency toward a transverse orientation. In cross walls of the same fungus, microfibrils were circularly arranged around a central pore. A similar arrangement of chitin microfibrils has been described in the septa of Chaetomium globosum (25).

Presence and content of chitin in fungi belonging to different taxonomical groups

As mentioned above, chitin is present in most fungi. Using microchemical tests, infrared spectrophotometry and x-ray diffraction, it has been shown to be present in the cell walls of selected species of most taxa: Chitridiomycetes, Hyphochytridiomycetes, Oomycetes, Zygomycetes, Deuteromycetes, Ascomycetes and Basidiomycetes. Chitin has not been detected in Schizomycetes, Myxomycetes and Trichomycetes.

It had been generally considered that Oomycetes lacked chitin in their cell walls, but x-ray diffraction studies by Lin and Aronson (22) revealed the presence of chitin and cellulose in the cell wall of Apodachlya sp. and more recently in the cell wall of the related species Leptomitus lacteus (23). These fungi contain in their cytoplasm unique granules, cellulin granules (28), which are composed of glucan and chitin (20). By x-ray diffraction, chitin and cellulose have been found also to co-exist in the cell wall of the hyphochytridiomycete Rhizidiomyces sp. (14) and in the ascomycete Ceratocystis ulmi (32). By use of infrared spectrophotometry it was found that the deuteromycete Epicoccum sp. possibly

contained both chitin and cellulose (26). This result has not been corroborated by use of x-ray diffraction.

Quantitative data of the content of glucosamine in the cell walls of fungi belonging to different taxa are shown in Table 2. Data were calculated from the tables recapitulated by Bartnicki-Garcia (4), and were completed with data that appeared in the more recent literature. Some data were originally reported as glucosamine and other as N-acetyl glucosamine. In general, they are regarded as a reflection of the amount of chitin present in the cell walls. Data are expressed as the mean of % glucosamine in the several species examined. Standard deviation and the lowest and highest values reported are included. Mode was calculated only for those groups which had enough representatives to give meaningful results and where data showed modal distribution. Fractional data were taken to the closest integer to calculate mode.

TABLE 2

GLUCOSAMINE CONTENT OF CELL WALLS FROM FUNGI BELONGING TO DIFFERENT TAXONOMIC GROUPS

TAXONOMIC GROUP	GLUCOSAMINE CONTENT (%)				
	MEAN	ST. DEVIATION	LOWER	HIGHER	MODE
Oomycetes	2.3	4.3	0.1	18.2	2
Chytridiomycetes	58.0	-	-	-	-
Zygomycetes	15.0	10.1	2.1	31.0	-
Hemiascomycetes	1.5	0.9	0.05	2.9	1
Euascomycetes	17.6	10.6	5.1	48.0	7.12
Loculoascomycetes	14.8	10.0	4.8	38.0	-
Homobasidiomycetes	12.4	16.7	1.6	61.0	8
Heterobasidiomycetes	4.5	-	3.7	5.4	-
Deuteromycetes	10.5	9.6	0.35	29.7	-

In the case of Oomycetes, the small amounts of glucosamine detected in the cell walls are not supposed to be present in chitin. Only the higher value (18.2%) which corresponds to Apodachlya sp. is considered to be chitin (23).

The only chytridiomycete reported, Allomyces macrogynus (1), contains one of the highest values of chitin, but it may not be representative of the whole class. As a group, Euascomycetes are the fungi with the highest content of chitin. The lowest value (5.1%) corresponds to a particular strain of Neurospora crassa (24); however, other strains of N. crassa have as much as 17.7% chitin in the wall. The highest value reported corresponds to Aspergillus oryzae. Hemiascomycetes are the taxonomical group that contains the lowest amounts of chitin in the cell wall, and,as mentioned above, it is restricted to the bud scars. The lowest value reported is from Hanseniospora uvarum, and the highest value from Saccharomycopsis

gutulata (4). Zygomycetes and Loculoascomycetes contain similar amounts of chitin. The lowest value for Zygomycetes corresponds to conidia from Mucor rouxii (3) and the highest value to the sporangiophore from Phycomyces blakesleeanus (27). Zygomycetes contain chitosan besides chitin in their cell walls. Since chitosan is solubilized by hot acid, the reported values of glucosamine correspond to that released by hydrolysis of the acid-insoluble residue. The lowest value for Loculoascomycetes was reported for the marine fungus Leptosphaeria albopunctata (39), and the highest value corresponds to the phytopathogen Cochliobolus miyabeanus (4).

Homobasidiomycetes contain lower amounts of chitin than Zygomycetes and Loculoascomycetes, but a minor number of genera have been analyzed. Lowest and highest values reported correspond to the same species Selerotium rolfsii, the lowest being for sclerotia and the highest for mycelium (4).

Even more minor amounts of chitin are present in the cell walls of Deuteromycetes; the lowest value was reported for Candida utilis and the highest value for Epidermophyton floccosum (4).

The only member of Heterobasidiomycetes whose cell wall has been analyzed is Tremella mesenterica (30). The yeast form contains lower amounts of chitin than the conjugation tube (see below).

From the collected data it is evident that there are large fluctuations in the chemical composition of the cell wall from different fungi belonging to the same taxonomical group, and even of different strains of the same species. A criticism which can be made of the analyses reported is that fungi were grown under different conditions, in media of variable composition, and for different periods of incubation. The cell wall cannot be considered as a static structure, but it may suffer gross changes in its composition depending on the conditions of growth. It has been demonstrated that synthesis of chitin continues after protein synthesis has been blocked by addition of cycloheximide (38).

Contents of chitin in spores and mycelia

There are only a few reports where the composition of cell walls from conidia has been compared with that from the mycelium or the sporophore. Collected data are shown in Table 3. With one exception, spores contain lower amounts of chitin than mycelium. The most striking differences correspond to Mucor rouxii, whose spores contain only 2% chitin compared to 9% of the mycelium and 18% of the sporophore, and particularly Trichoderma viride whose conidia have no chitin, whereas the mycelium contains 12-22% chitin depending on the age of the culture (7).

TABLE 3

RELATIVE AMOUNTS OF CHITIN IN THE CELL WALL OF FUNGI AT DIFFERENT STAGES

FUNGUS	STAGE	CHITIN (%)
Mucor rouxii	Mycelium	9.4
	Sporangiophore	18.0
	Sporangiospore	2.1
Aspergillus phoenicis	Mycelium	23.7
	Conidia	36.2
Neurospora crassa	Mycelium	8.0-11.9
	Conidia	7.4-9.0
Penicillium chrysogenum	Mycelium	19.5-42
	Conidia	11.4
Trichoderma viride	Mycelium	12-22
	Conidia	0

Comparative amounts of chitin in the cell walls of dimorphic fungi

There are fungi which show different morphology when grown under different conditions. They are called dimorphic fungi. Cell walls from the yeast and mycelial forms of several dimorphic fungi have been analyzed, and the results are summarized in Table 4.

TABLE 4

RELATIVE AMOUNTS OF CHITIN IN THE CELL WALL OF SOME DIMORPHIC FUNGI

FUNGUS	MORPHOLOGICAL STAGE	CHITIN (%)
Mucor rouxii	Mycelium	9.4
	Yeast	8.4
Saccharomycopsis gutulata	Mycelium	2.3
	Yeast	1.7
Blastomyces dermatitidis	Mycelium	13
	Yeast	44
Histoplasma capsulatum	Mycelium	4, 25.8
	Yeast	25, 11.5
Histoplasma farciminosum	Mycelium	40
	Yeast	25.8
Paracoccidioides brasiliensis	Mycelium	11
	Yeast	37
Tremella mesenterica	Yeast	3.7
	Conjugation tube	5.4

There is no correlation between the morphology of the organism and the content of chitin. Mucor rouxii, Saccharomycopsis gutulata and Histoplasma farciminosum contain slightly higher amounts of chitin in the mycelial form than in the yeast form; whereas Paracoccidioides brasiliensis and Blastomyces dermatitidis contain more chitin in the cell wall of the yeast form. Conflicting reports exist on the chemical composition of the cell wall from Histoplasma capsulatum; whereas Domer et al.(12) reported that the yeast form contained more chitin than the mycelial form (25% vs. 4%). Kanetsuna et al.(18) reported that the mycelial form contained 25.8% chitin and the yeast form only 11.5%. Interestingly, the related species Histoplasma farciminosum (36) is the dimorphic fungus which contains the highest amount of chitin in the mycelial form.

Included in Table 4 are data on the composition of the cell wall from the basidiomycete Tremella mesenterica. This organism grows in a yeast-like form, but a long conjugation tube is induced by the presence of specific hormones (2). Analyses of the cell walls from the yeast and the conjugation tubes revealed that the latter contained larger amounts of chitin (30).

From the data presented in Table 4, it becomes apparent that the mere difference in the relative amounts of chitin in the cell wall cannot explain the difference in morphology. Cell shape is probably determined by the pattern of growth of the cell wall. It has been shown (5) that filaments grow only in the apical region, whereas yeasts grow uniformly throughout the cell surface. Alternatively (or simultaneously), differences in the regulation of chitin synthetase may explain the pattern of wall construction (34).

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THE DETECTION AND ESTIMATION OF CHITIN IN INSECT ORGANS

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ABSTRACT

Chitinous membranes are never pure in nature. Usually some chemical purification is necessary before applying a test, and for small and delicate parts the purification is of uncertain validity. Several techniques that are valid with robust samples (e.g., x-ray diffraction) cannot be applied to tiny delicate objects in a heterogeneous medium.

The presentation will cover the working definitions used by various workers. These include (1) simply weighing the residue remaining after prolonged extraction with NaOH; (2) determining the amount of glucosamine or acetylglucosamine after acid or enzymatic hydrolysis of such residues from extraction with alkali; (3) the incorporation of C^{14} labeled glucosamine or acetylglucosamine into an alkali-insoluble component; (4) the classical van Wisselingh chitosan color test with iodine; and (5) the fluorescent chitinase reaction.

INTRODUCTION

My remarks will be of little interest to those whose concern with Arthropods is only as the raw material for the manufacture of chitin and chitosan. As a biologist, I am interested in questions such as: Are all membranes that are dispersed by hot alkali really devoid of chitin? Do chitin percentages range in a continuous series from 60 or 80% down to zero? And, if similar appearing membranes occur with and without chitin, then what is the significance of chitin? These questions cannot be definitely answered today. How close can we come?

METHODS

No pure samples of chitin are known to occur naturally, and to judge from data such as Rudall's x-ray diffraction studies, chitin does not occur free in cuticles but only in association with protein to which it is bound in some manner. In practice, chitin is a substance that is not dispersed by hot 1 N NaOH. Further characterization may or may not be done. The residue from alkaline purification may be quantified by either gravimetric determinations or by estimation of glucosamine (GA) or acetylglucosamine (AGA) following acid or enzymatic hydrolysis. By definition, then, any units which are removed during 'purification' would not be called chitin. The inadequacy of this conclusion will be dealt with in discussing the van Wisselingh chitosan color test.

In 1971 Hackman and Goldberg (2) proposed a semi-micro method of chitin

analysis. This involved dissecting off integuments, cleaning off the epidermal cells by swabbing in 70% ethyl alcohol, extracting with chloroform-methyl alcohol (2:1), drying, and powdering in a Wiley mill. The powder was extracted 2X with 1 N NaOH at 100° for 24 hours, and the supernatants discarded. Without removing the residue from the tube, it was washed successively with water, 2 N HCl to remove adsorbed alkali, 6X with ethyl alcohol and 3X with diethyl ether. Tubes with residues were dried and weighed. Then the 'chitin' was removed and the tube washed and re-weighed. Stated values for chitin were, then, the difference in weight of tube + chitin minus tube alone. Using the above method, the authors concluded that the cuticles of ticks contained 3.8% chitin and the membranes of a bloodsucking bug 11.2%. While the authors did not state it, this technique assumes that all chitin is insoluble in 1 N NaOH and none disperses in any way to become lost (remember that the supernatants were discarded); it also assumes that nothing else is insoluble in 1 N NaOH. Clearly, the method does not employ balance-sheet procedures that account for all components.

For the peculiar 'living fossil' Peripatus, Hackman gave a value of 8% chitin in the cuticle based on calculations from the GA content of acid hydrolysates of alkali-treated cuticles. Many more such values have been given by Jeuniaux from enzymatic hydrolysates of alkali-treated cuticles. Jeuniaux obtained lower values, sometimes much lower values, when the cuticles were not first 'purified' by treatment with alkali. This is the genesis of Jeuniaux's concepts of 'free' and 'bound' chitin which explains higher values from alkali-treated cuticles as being due to the removal of masking by certain chitin-protein associations. GA and AGA are found in the hydrolysates of numerous things other than chitin, but the tacit assumption is made that such alternative sources of GA and AGA have all been removed by treatment with hot 1 N NaOH. Again we encounter the idea that chitin is immune to alkali which, however, allegedly removes everything else (exceptions can be cited for both points in this sentence).

The incorporation of C^{14} labeled GA or AGA into an alkali-insoluble fraction has been reported. While this can be a good approach for studying chitin deposition, it does not seem useful for the identification of chitin as it appears in naturally occurring structures.

This leads to a consideration of the time-honored van Wisselingh chitosan color test. This test involves partial or complete deacetylation of chitin with hot conc KOH to produce chitosan. The more or less deacetylated chitin reacts with iodine at low pH to give a characteristic violet color. Since no other compound resistant to hot concentrated alkali is known to give this color reaction, it is considered specific. Even though the test seems to be reliable when positive, it is still dubious in terms of universal applicability.

In the first place, any object being tested must still be recognizable after heating in conc KOH for some minutes. Since such drastic treatment with alkali destroys most biological structures, the object being tested must not only be durable, but also sufficiently large to be found and identified. Thus, tracheoles (the terminal branches of the respiratory tree in insects) are said to be dispersed, but their size is such that one wonders if they are really dispersed or just no longer recognizable.

More obvious is the case of delicate structures that disintegrate under the normal van Wisselingh procedure, but which if treated less violently

may survive to give a positive chitosan color test. The minute scales on the wings of butterflies and moths are good examples. In many species these contain enough chitin to survive the most violent alkaline treatment. In other species some of the scales will only survive a less hot, less concentrated or less prolonged treatment--and then give a positive color test. Scales of a very few species never survive treatment with hot alkali. Results with scales of more than 100 species are tabulated by Richards (5). These homologous structures from different species of this group of insects form a series in which some give strongly positive test results and hence may be presumed to contain a considerable percentage of chitin. Others contain either less or more readily dispersed chitin, and some seem to be negative--whatever invariable dissolution in hot alkaline solutions means.

The results from these tests, reported 30 years ago, seem clear. Something giving the van Wisselingh color test for chitin can be dispersed by alkali. What, then, is the validity of chitin determinations based simply on alkali-insoluble residues, or GA or AGA determinations from such alkali-insoluble residues? As a biologist, perhaps I can be excused for asking why no qualified chemist has addressed himself to the problem of what is dispersed from such preparations by alkali. Chitins of various molecular weight, such as described by Strout, Lipke and Geoghegan, may be part of the answer; we do not know.

One would think that there must be some minimal concentration of chitin for a structure to remain recognizable after removal of other components. Most chitin-containing integuments are reported to contain 30-40% chitin on a dry-weight basis (3, 6). A few insect species have values as high as 60% (some decapod Crustacea have values up to 85%, if one considers only the organic components), but a few are recorded as having only 2-4%. These low values have sometimes been induced only by gravimetric determinations of the amount of alkali-insoluble residue, but in other cases, they are calculated from AGA content. It would indeed be interesting to know if the component called chitin in these cases with low percentages was really the same as chitin present in high proportions. I wonder if the determinations resulting in reporting such very low percentages are valid. I do not know of any case where a report of very low percentage has been accompanied by identification of all that was removed.

With the uncertainties just itemized, I welcomed the report of the development by Benjaminson (1) of a fluorescent-enzyme technique that seems to hold great promise. He conjugated chitinase with either fluorescein isothiocyanate or lissamine rhodamine B 200 chloride and took advantage of the attachment of an enzyme to its substrate to localize chitin by fluorescence microscopy. This method is comparable to the powerful fluorescent-antibody technique that has been so valuable in immunology. However, the description indicated that only a minimal amount of testing had been done. Benjaminson showed only that the method could work. We bought some chitinase from a commercial source and prepared the fluorescent enzyme only to find that the chitinase was so impure that many things were stained. We attempted to purify the enzyme, and did clean it up by column chromatography to the point that other things were only faintly stained when known chitinous structures were intensely stained. Chemists at the laboratory where Marks & Leopold (4) work did a better job, and nice looking pictures resulted (Figs. 1-6). This technique does not have the ambiguity arising from a preceding treatment with alkali.

There has still been inadequate testing of the fluorescent-enzyme method.

The above authors used only normal cuticles that were not of great delicacy. Presumably these contained the usual concentration of α-chitin. Tests have not yet been made to check the method with all the known crystallographic types of chitin (α, β and γ), with low as well as high molecular weight chitins, with 'free' versus 'bound' chitin (3), with chitosan, with dispersed and regenerated chitins and chitosans, or with membranes which are dispersed by alkali (tracheoles, epicuticle, air sacs of bees, etc.). Presumably, like histochemical methods in general, the fluorescent-enzyme method will be a qualitative test not readily made quantitative. However, qualitative tests are good for identification and localization.

In conclusion, numerous real advances in chitin chemistry have been made in recent decades, but the ability to identify chitin with certainty in small and delicate structures has advanced little. In the 8 years since it was described, the fluorescent-chitinase technique has been used by only one set of authors. It has real promise but needs extensive evaluation.

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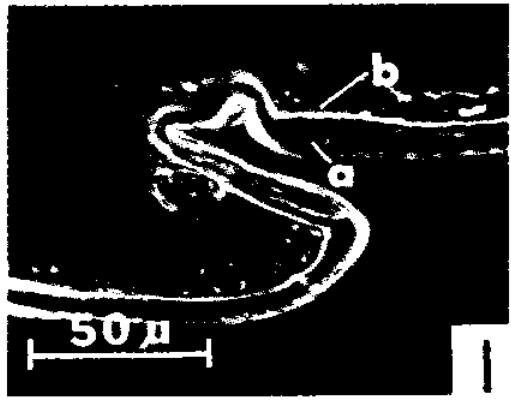

Figure 1. A section through the cuticle of a leg of a freshly molted cockroach as seen in a phase-contrast microscope. a = cuticle; b = epidermis. All photographs from Marks & Leopold (4).

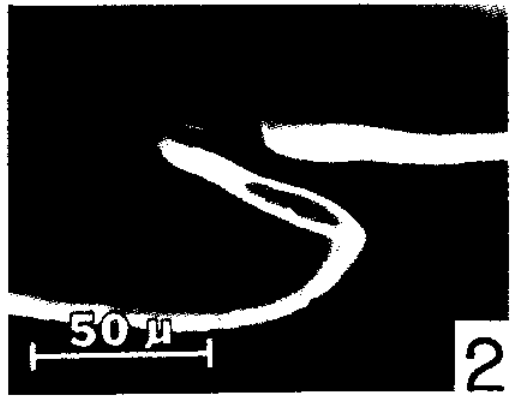

Figure 2. Same specimen stained with fluorescent-chitinase and observed in a fluorescent microscope. Note that only the cuticle is stained.

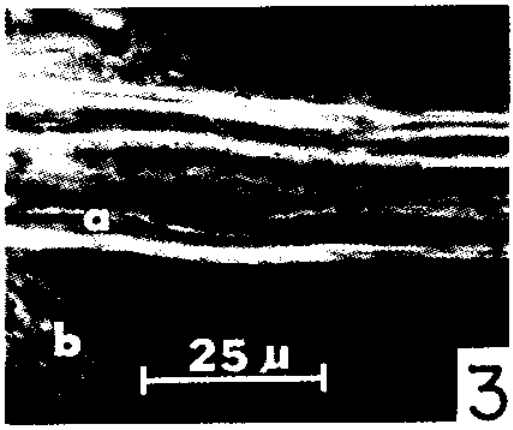

Figure 3. Section through the cuticle of a regenerating leg at 25 days in vitro as seen in a phase-contrast microscope. a = cuticle; b = epidermis.

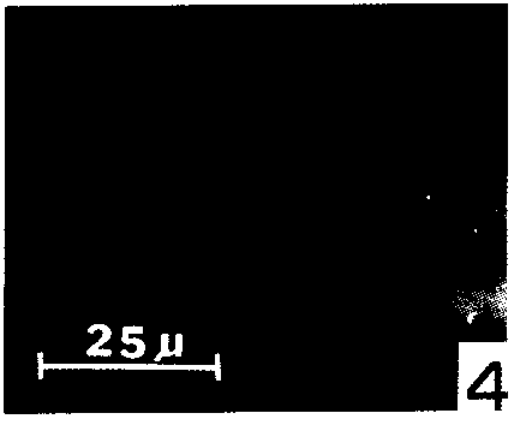

Figure 4. Same specimen stained with fluorescent-chitinase and observed in a fluorescent microscope. Note low intensity of fluoresence in contrast to the normal leg (Fig. 2).

Figure 5. Section through the cuticle of a regenerating leg at 10 days in vitro as seen in a phase-contrast microscope. a = refractile droplets; b = cuticle; c = epidermis.

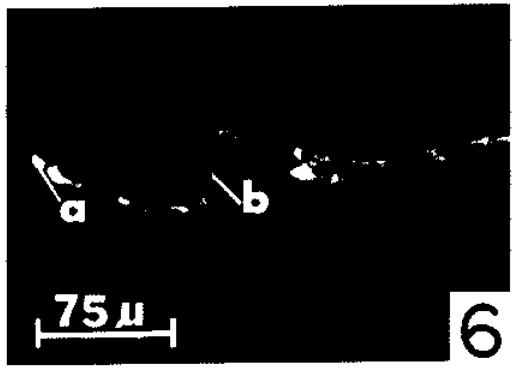

Figure 6. Same specimen stained with fluorescent-chitinase and observed in a fluorescent microscope. The thin cuticle (b) appears as a bright line; the refractile droplets (a) between cuticle and epidermis are also fluorescent and hence assumed to contain chitin.